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The plasma membrane ATPase of *Dictyostelium discoideum*

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During centrifugation of *Dictyostelium* membranes on sucrose and metrizamide gradients, an ATPase activity resistant to azide and molybdate but sensitive to diethylstilbestrol was found to copurify with the plasma membrane markers alkaline phosphatase and ^{125}I in cells surface-labelled by lactoperoxidase catalyzed iodination. This ATPase was enriched 50-fold in purified plasma membranes and could be separated from the mitochondrial ATPase on metrizamide gradients. The plasma membrane ATPase is very specific for ATP as substrate and Mg^{2+} as essential cofactor. Its pH optimum is 6.5 and it is inhibited by dicyclohexylcarbodiimide, diethylstilbestrol, vanadate, mercurials and Cu^{2+}, but not by ouabain, molybdate, azide or oligomycin. It was not specifically affected by either monovalent cations or anions. These results suggest that the plasma membranes of *Dictyostelium* contain an ATPase similar to the proton-pumping ATPases recently identified in fungal and plant plasma membranes (Serrano, R. (1984) Curr. Top. Cell. Regul. 23, 87–126).

Introduction

The cellular slime mould *Dictyostelium discoideum* is a model system in differentiation studies, where cAMP induces the aggregation of amoeba and their differentiation into both stalk and spore cells [1]. Recent experimental evidence suggests that the choice between both differentiation pathways may be mediated by changes in the intracellular pH [2]. Weak acids and acidic buffers induce stalk cell formation, while weak bases (ammonia) and alkaline buffers favour spore formation. A low molecular-weight substance has been purified from amoebal cultures and shown to induce stalk cell formatoin [3]. Similar effects are obtained with the ATPase inhibitor diethylstilbestrol, and therefore it has been proposed that the natural morphogen could act by inhibiting a proton-pumping ATPase, and therefore causing intracellular acidification [2].

A novel type of ATPase has recently been identified in fungal and plant plasma membranes [4]. This enzyme operates as an electrogenic proton pump and forms a phosphorylated intermediate in a major subunit of 100 kDa. It is inhibited by vanadate, diethylstilbestrol and dicyclohexylcarbodiimide, but insensitive to oligomycin, azide or ouabain. This plasma membrane ATPase plays a central role in fungal and plant physiology, because it generates an electrochemical proton gradient which drives active nutrient transport, and it also regulates intracellular and extracellular pH.

Several phosphatase activities have been de-

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Abbreviations Mes, 4-morpholineethanesulphonic acid, metrizamide, 2-(3-acetamido-5-*N*-methylacetamido-2,4,6-triiodobenzamido)-2-deoxy-D-glucose.

scribed in the plasma membranes of *Dictyostelium* [5], but they are 'ectoenzymes', acting on extracellular phosphate esters, and are therefore not related to the fungal and plant plasma membrane ATPases referred to above. This type of ATPase is described here in *Dictyostelium* for the first time. The identification of this likely proton pump could facilitate future studies on the role of proton fluxes and changes in intracellular pH in the differentiation of *Dictyostelium*.

Methods

Strains and growth conditions

Dictyostelium discoideum, strain AX-2, was grown axenically as described [6]. Cells were harvested during late exponential phase at $(4–5) \cdot 10^6$/ml and washed twice with water by centrifugation during 5 min at $500 \times g$.

Plasma membrane preparation

All operations were carried out at 0–4°C and the sucrose, glycerol and metrizamide solutions contained 10 mM *N*-tris-(hydroxymethyl)methylglycine adjusted to pH 7.5 with Tris/0.1 mM EDTA/0.1 mM dithioerytritol. Relative centrifuge forces refer to the average radius of the tubes.

The cells were resuspended at $(1–2) \cdot 10^8$/ml in a medium with 6% sucrose/50 mM Tris (pH 8.5 with HCl)/5 mM EDTA/1 mM dithioerytritol. Homogenization was performed by shaking with two volumes of glass beads (0.5 mm) during 2 min. Small volumes of cell suspensions (1–5 ml) were shaked manually in a cyclo-mixer (Super-Mixer, Lab-Line Instruments, Melrose Park, IL, U.S.A.). Large volumes were processed in 90-ml batches with a Vibrogen Cell Mill (E. Bühler, Tübingen, F.R.G.). The glass beads were removed by filtration and washed with one volume of 20% glycerol (v/v). The filtrate was centrifuged during 5 min at $800 \times g$, and the supernatant is referred to hereafter as the homogenate.

The homogenate was centrifuged during 20 min at $24\,000 \times g$ and the supernatant was discarded. The pellet (referred to as crude membranes) was resuspended at 5–10 mg protein per ml with 20% glycerol and stored at −20°C until further processing. Discontinuous sucrose gradients were prepared by layering 4 ml 32% sucrose (w/w) over 4 ml 43% sucrose (w/w). After layering 4 ml of crude membranes, the tubes were centrifuged during 3 h at $200\,000 \times g$ in a Beckman SW 40 Ti rotor. The 32/43% interphase was collected, diluted with three volumes of water and centrifuged during 20 min at $90\,000 \times g$. The pellet was resuspended at 1–3 mg protein per ml with 20% glycerol and stored at −20°C until further processing. This consists on layering 1 ml over a cushion of 3 ml 25% metrizamide (w/v) and centrifuging during 2 h at $250\,000 \times g$ in a Beckman SW 50.1 rotor. The glycerol/metrizamide interphase was collected, diluted with water and centrifuged as above. The pellet was resuspended at 0.5–1 mg protein per ml with 20% glycerol and stored at −20°C.

Centrifugation on continuous gradients

Linear gradients of sucrose and metrizamide were prepared by the simplified method of Stone [7]. In the case of sucrose gradients three layers with 5 ml of 46, 38 and 25% sucrose (w/w) were employed and for metrizamide gradients three layers with 3.5 ml of 34, 26 and 17% metrizamide (w/v) were used. The tubes were capped, placed in a horizontal position and allowed to diffuse for 3 h. After centrifugation as indicated in the legend of the figures, fractions were collected from the top with a Buchler auto-densi flow apparatus.

Enzyme activities

ATPase activity was measured at 30°C in 1 ml of medium with 50 mM Mes-Tris (pH 6.5)/5 mM $MgSO_4$ and, when indicated, 0.2 mM ammonium molybdate/5 mM sodium azide/0.15 mM diethylstilbestrol. The reaction was started with 2 mM ATP and the P_i liberated in 30 min measured as described in **Ref. 8**.

Alkaline phosphatase was measured at 30°C in 1 ml of medium with 50 mM Tris-Mes (pH 9.0)/5 mM $MgSO_4$. The reaction was started with 2 mM *p*-nitrophenylphosphate and the P_i liberated in 60 min determined as described above.

1 unit of phosphatase activity corresponded to 1 μmol of P_i liberated per min.

Cytochrome *c* oxidase was measured as described [9] and 1 unit of activity corresponded to 1 μmol of cytochrome *c* oxidized per min.

Measurement of protein concentration

The method of Bradford [10] was modified as described by Read and Northcote [11], and bovine serum albumin has been served as standard.

Chemicals

The following products were obtained from Sigma: ATP (sodium salt, grade I), ADP (grade I), AMP (type II), GTP (type I), UTP (type I), ITP, CTP, IDP, UDP, diethylstilbestrol, *p*-chloromercuriphenylsulfonate, *p*-nitrophenylphosphate (sodium salt), cytochrome *c* (type III) and metrizamide (grade I). Dicyclohexylcarbodiimide was from Merck and sodium orthovanadate from Fisher. Dicyclohexylcarbodiimide and diethylstilbestrol were dissolved in methanol and the small amounts of this solvent introduced in the assays (less than 1%) has no effect on the ATPase activity.

Results and Discussion

The Mg^{2+}-ATPase activity resistant to azide and molybdate and sensitive to diethylstilbestrol has been proposed as marker for fungal and plant plasma membranes [4]. The fate of this activity during fractionation of *Dictyostelium* homogenates has been compared with that of alkaline phosphatase, cytochrome oxidase and ^{125}I in cells surface-labelled by lactoperoxidase catalyzed iodination. Alkaline phosphatase has been widely utilized as marker for the *Dictyostelium* plasma membrane [5,12,13] and surface-labelling with ^{125}I has been utilized to tag the plasma membrane of different cells [14] including *Dictyostelium* [6,12]. Cytochrome oxidase is a classical mitochondrial marker. About 80% of the putative plasma membrane ATPase and cytochrome oxidase activities, together with 50% of alkaline phosphatase and 60% of radioactivity were recovered by sedimentation at $24000 \times g$ and these crude membranes were fractionated by isopycnic centrifugation on continuous sucrose gradients (Figs. 1 and 2). The peak of ATPase was centered around 35% sucrose (w/w, density 1.15) and was mostly coincident with the peak of radioactivity (Fig. 2). On the other hand, alkaline phosphatase was distributed in two peaks, one coincident with the ATPase and other at lower density (Fig. 1). This is in accordance with the suggestion of a dual localization of the enzyme in *Dictyostelium* [5].

Although sucrose gradient centrifugation has been previously employed for the purification of *Dictyostelium* plasma membranes [12,13,15], we could not achieve separation of mitochondrial and plasma membrane markers in these gradients (Figs. 1 and 2). Therefore, the fractions enriched in

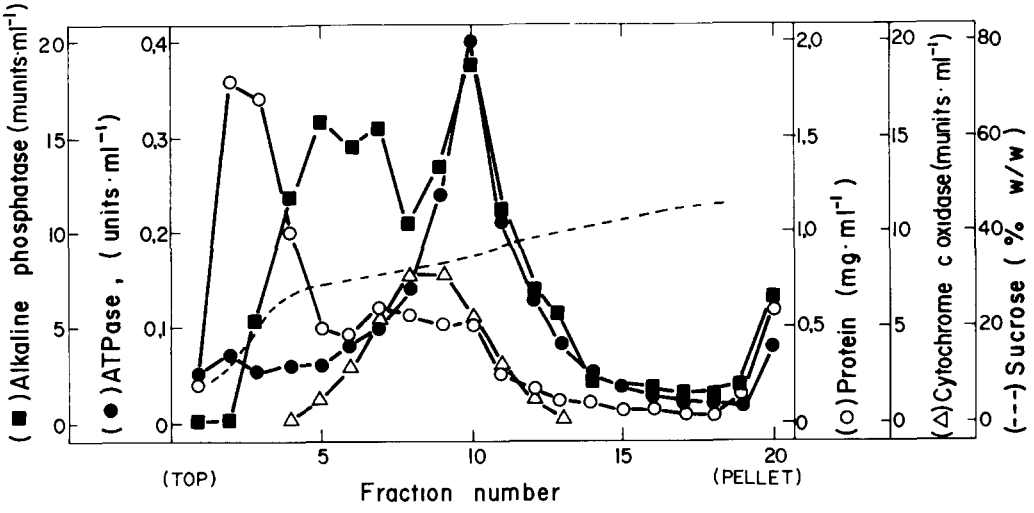

Fig 1. Purification of membranes by sucrose gradient centrifugation Crude membranes (12 mg protein in 2 ml) were applied to 15 ml of a continuous sucrose gradient and centrifuged during 14 h at $50000 \times g$ in a Beckman SW 27 rotor. The distribution of ATPase (measured in the presence of azide and molybdate) (●), alkaline phosphatase (■), cytochrome *c* oxidase (△) and protein (○) in the different fractions is shown.

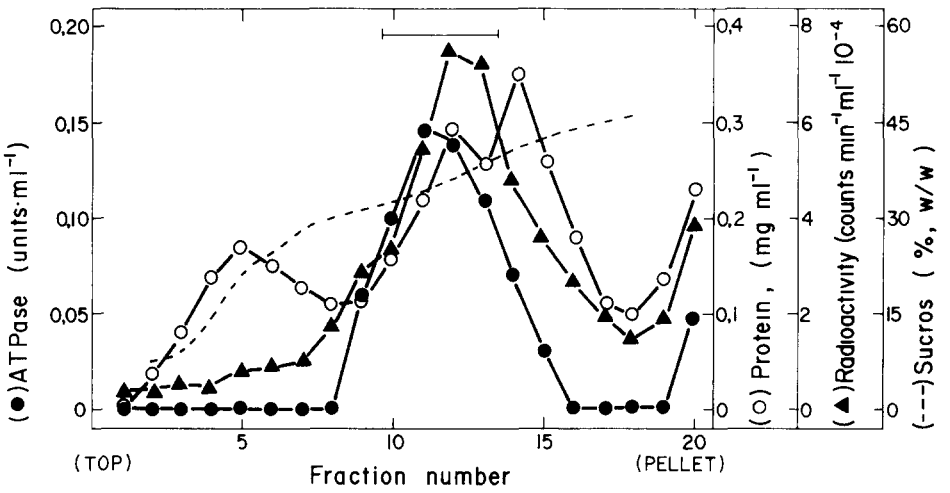

Fig 2 Distribution of ATPase (measured in the presence of azide and molybdate) (●), radioactivity (▲) and protein (○) during sucrose gradient centrifugation. Conditions as in Fig 1, except that the cells were surface-labelled with ^{125}I before homogenization and 6.6 mg protein of crude membranes were applied to the gradient

plasma membranes from the sucrose gradients were further purified by centrifugation on metrizamide gradients. Biological particles exhibit lower densities in metrizamide than in sucrose, and this shift may differentiate particles exhibiting similar densities in sucrose [16]. As indicated in Figs 3 and 4, radioactivity, ATPase and alkaline phosphatase showed a coincident distribution on metrizamide gradients, while the cytochrome oxidase equilibrated at much higher densities. Plasma membranes were distributed in variable proportions between two peaks of low density. No significative differences in enzyme activities or polypeptide composition were observed between both peaks, and a likely explanation is that they correspond to membranes with different permeability to metrizamide.

The parallelism in the distribution of ATPase,

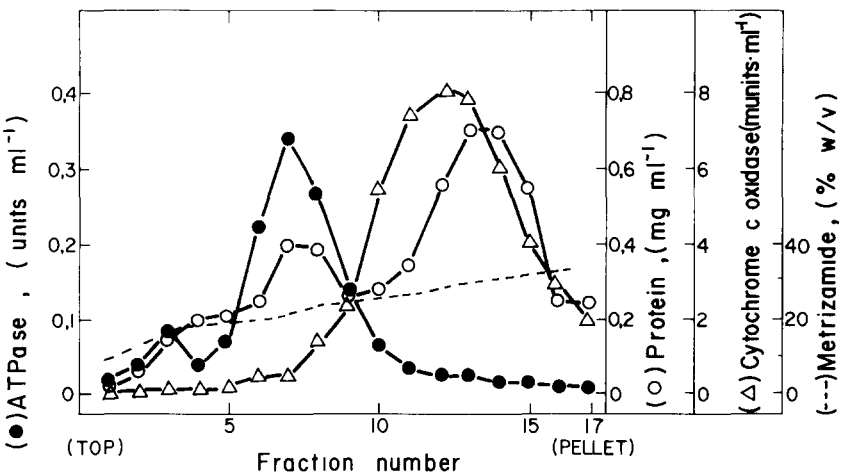

Fig 3 Further purification of membranes from sucrose gradients on metrizamide gradients 2.7 mg protein of membranes equilibrating at 32–43% sucrose (see Fig. 1) were resuspended in 20% glycerol (1 5 ml) and applied to 10 5 ml of a continuous metrizamide gradient which was centrifuged during 2 5 h at 150000 × *g* in a Beckman SW 40 Ti rotor Symbols as in Figs 1 and 2

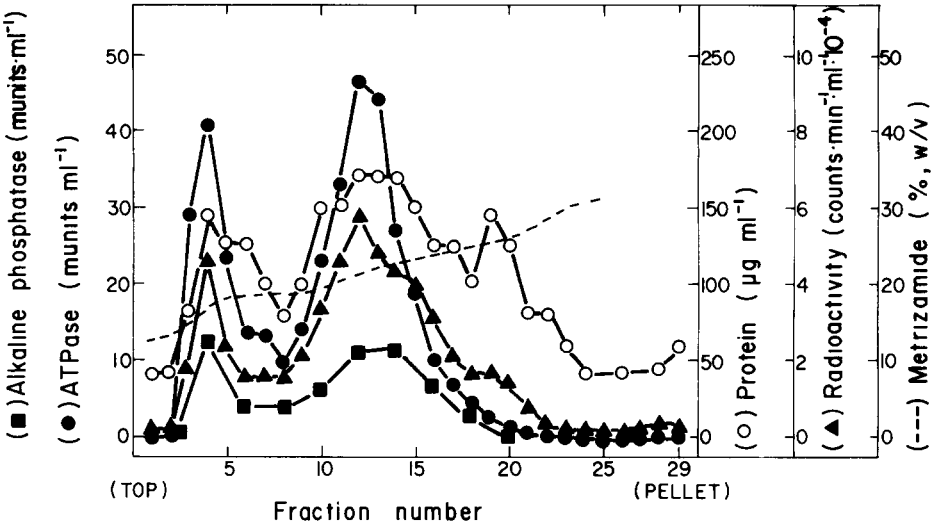

Fig 4 Distribution of ATPase (measured in the presence of azide and molybdate) (●), radioactivity (▲), alkaline phosphatase (■) and protein (○) during metrizamide gradient centrifugation Conditions as in Fig. 3, except that the sample was 1.2 mg protein of membranes equilibrating at 31–39% sucrose in the gradient of Fig 2

radioactivity and alkaline phosphatase suggest that the ATPase is a plasma membrane enzyme. A variable degree of inactivation of the ATPase (from 40 to 70%) occurred during centrifugation on continuous metrizamide gradients, suggesting that the metrizamide may be somewhat deleterious to the enzyme. Even greater inactivations were observed with the plasma membrane ATPase of oat roots (Serrano, R., unpublished data). This inactivation does not occur when the continuous metrizamide gradient was substituted by a cushion of 25% metrizamide, which could not be crossed by the plasma membrane but allowed the passage of mitochondria and other contaminants (see Methods).

As indicated in Table I, the ATPase was enriched 50-fold in the final plasma membrane preparation, which was virtually free of mitochondrial contamination. Alkaline phosphatase was enriched 30-fold, in accordance with the suggestion of a dual localization of this enzyme. The enrichment of radioactivity was only 15–20-fold (data not shown). This lower enrichment can be explained by the observation that about 40% of the radioac-

TABLE I

SPECIFIC ACTIVITY OF MARKER ENZYMES DURING PURIFICATION OF PLASMA MEMBRANES

The meaning of the fractions is defined in Methods. Protein refers to the fractionation of 1 10^{10} cells. The plasma membrane ATPase is defined as the activity resistant to azide and molybdate, but sensitive to diethylstilbestrol. The last inhibitor blocked 30–50% of the activity in the homogenate, 50–70% in the crude membranes and over 80% in the sucrose and metrizamide gradients. Results are the mean of three experiments.

Fraction	Protein (mg)	Plasma membrane ATPase (milliunits per mg protein)	Alkaline phosphatase (milliunits per mg protein)	Cytochrome *c* oxidase (milliunits per mg protein)
Homogenate	550	25	2.5	1 5
Crude membranes	70	163	14	6
Scurose gradient	7	820	40	14
Metrizamide gradient	4	1 300	70	<1

tivity of the homogenate was present in soluble proteins, suggesting some degree of cells lysis during the labelling procedure. In addition, as indicated in Fig. 2, some radioactivity was present in structures of higher density than the plasma membrane. These may correspond to carbohydrate-rich material present in the cell surface, as recently described for yeast protoplasts [17]. The yield of protein and ATPase in the plasma membrane preparation were 0.7 and 38%, respectively. Therefore, it can be calculated that the *Dictyostelium* plasma membrane represents about 1.8% of cellular protein. This value is slightly lower than previous estimations in preparations purified only by sucrose gradients [12,13,15].

As indicated in Fig. 5, the plasma membrane ATPase was sensitive to vanadate, diethylstilbestrol and dicyclohexylcarbodiimide, with half maximal inhibition at 12, 60 and 46 μM, respectively. In addition, the enzyme was very sensitive to the sulfhydryl group reagents Cu^{2+} and *p*-chloromercuryphenylsulfonate, which produced 50% inhibition at 10 μM. No inhibition was observed with either oligomycin (10 μg/ml), azide (5 mM), ammonium molybdate (0.2 mM) or ouabain (0.5 mM).

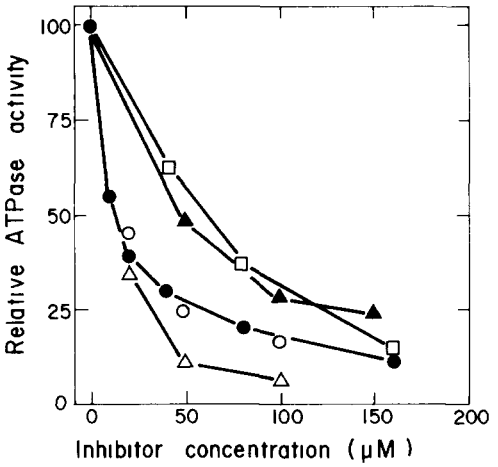

Fig 5. Effect of vanadate (●), diethylstilbestrol (□), dicyclohexylcarbodiimide (▲), $CuSO_4$ (△) and *p*-chloromercuriphenylsulfonate (○) on the ATPase activity of plasma membranes purified by sucrose and metrizamide gradients The assays contained 9 μg ml^{-1} protein and the indicated concentrations of inhibitors. The reaction was started with ATP (1 mM) after 5 min preincubation. The absolute activity in the absence of inhibitors was 1.0 units per mg protein.

The pH optimum was 6.5, with 50% reduction in activity at pH 5.3 and 7.6 (Fig. 6). In membranes purified only by sucrose gradients the relative activities at acid and alkaline pH were much greater, reflecting contamination by acid phosphatase (sensitive to molybdate) and mitochondrial ATPase (sensitive to azide, Fig. 6).

In metrizamide-purified plasma membranes, the phosphatase activity measured at pH 6.5 in the presence of Mg^{2+} is very specific for ATP (Table II), other nucleotides and phospahte esters being hydrolyzed with rates less than 20% of ATP. The membranes contained a non-specific phosphatase, assayed with *p*-nitrophenylphosphate, with similar activities at acid and alkaline pH. However, at pH 5 molybdate inhibited 90% of the activity, while at pH 9 it only produced 20% inhibition. This suggest the presence of both acid and alkaline phosphatases, only the first one sensitive to molybdate. The hydrolysis of *p*-nitrophenylphosphate at either pH 6.5 or 9 was not inhibited by diethylstilbestrol As this drug inhibits 90% of the ATPase activity (Fig. 5), it can be estimated that only 10% of the hydrolysis of ATP is due to non-specific phosphatases. The low activity observed with other nucleotide triphosphates (Table II) is only inhibited 50–60% by diethylstilbestrol, suggesting

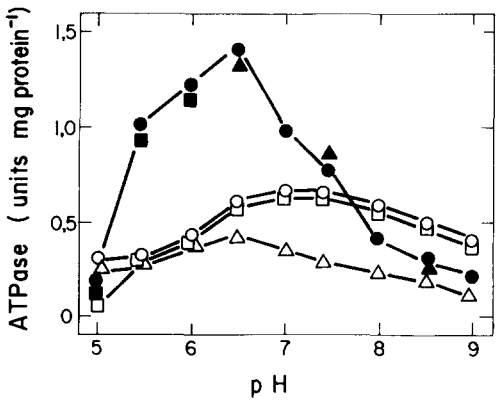

Fig 6 Effect of pH on the ATPase activity of plasma membranes purified by sucrose gradients (open symbols) and by both sucrose and metrizamide gradients (closed symbols). The assay medium contained 50 mM Tris-Mes buffers of the indicated pH, 5 mM $MgSO_4$ and 2 mM ATP Circles no inhibitor present, squares with 0.2 mM ammonium molybdate to inhibit acid phosphatase, triangles. with 5 mM sodium azide to inhibit mitochondrial ATPase

TABLE II

SUBSTRATE SPECIFICITY OF THE PLASMA MEMBRANE ATPASE

Plasma membranes were purified by sucrose and metrizamide gradients and the phosphatase activity assayed in a medium with 50 mM Mes-Tris (pH 6.5)/5 mM $MgSO_4$/0.6 mM substrate The absolute activity with ATP was 1 0 unit per mg protein Results are the mean of two experiments.

Substrate	Relative activity
ATP	100
ITP	20
UTP	20
GTP	18
CTP	12
p-Nitrophenylphosphate	10
PPi	9
ADP	7
IDP	7
AMP	4
UDP	2

that the activity of the ATPase on other nucleotides is less than 10% that with ATP.

The ATPase exhibited hyperbolic kinetics in the range from 0.05 to 2 mM ATP, with a K_m of about 0.1 mM. Mg^{2+} is required for ATPase activity, and in the absence of this cation ATP hydrolysis was reduced to less than 10%. Ca^{2+} and Mn^{2+} could partially substitute for Mg^{2+}, but Ca^{2+} was inactive. In addition, Ca^{2+} inhibited the activity measured in the presence of Mg^{2+} (86% inhibition at 4 mM). Salts of monovalent cations produced a small activation (10–20% at 20 mM), but the effect was not specific, with similar activities obtained with K^+, Na^+, choline$^+$ and Tris$^+$ as cations and Cl^-, acetate$^-$, SO_4^{2-} and NO_3^- as anions. No synergistic effects of Na^+ and K^+ were observed.

Preliminary experiments indicate that a plasma membrane polypeptide of about 100 kDa is transiently phosphorylated in the course of ATP hydrolysis, and therefore it probably corresponds to the catalytic subunit of the plasma membrane ATPase (data not shown).

These results indicate that the plasma membranes of *Dictyostelium* contain an ATPase with similar properties than the proton-pumping ATPases recently identified in fungal and plant plasma membranes [4]. It is likely that the *Dictyostelium* enzyme also operates as a proton pump, and therefore it could drive active transport coupled to the proton gradient and also regulate intracellular and extracellular pH. The identification of this enzyme will allow experimental tests of the hypothesis that *Dictyostelium* morphogens may act by inhibiting a proton pumping ATPase of the plasma membrane [2].

A recent report [18] suggest the presence on *Dictyostelium* plasma membranes of an ATPase activity sensitive to azide and stimulated by monovalent cations. This enzyme is clearly different from the plasma membrane ATPase described in the present work, which is affected neither by azide or monovalent cations. The inhibition of the former ATPase by azide, which is a typical inhibitor of bacterial and mitochondrial ATPases indicates that control experiments should discard the possibility of contaminatoin of the membranes employed in that work by these azide-sensitive enzymes.

Finally, the purification of *Dictyostelium* plasma membranes by a combination of sucrose and metrizamide gradients seems to provide a preparation of greater purity than previously described procedures. In addition, the ATPase activity resistant to azide and molybdate and sensitive to diethylstilbestrol seems to be the most convenient enzymatic marker for the *Dictyostelium* plasma membrane.

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